PHYSICOCHEMICAL CHARACTERIZATION, MICROBIOLOGICAL QUALITY CONTROL AND TOXICITY EVALUATION OF THE HIDROETHANOLIC EXTRACT FROM CHENOPODIUM AMBROSIOIDES LINN

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Keywords: Chenopodium ambrosioides, Amaranthaceae, physicochemical characterization, microbiological quality control, acute oral toxicity, cytotoxicity.

ABSTRACT: Chenopodium ambrosiodes Linn, member of the National List of Medicinal Plants of Interest to SUS (RENISUS), is used to treat different diseases and shows good potential to generate products of interest to the Brazilian Unified Health System “Sistema Único de Saúde” (SUS). However, the use of herbal derivatives implies the control of their quality and the evaluation of their toxicity. Pharmacopeial tests were performed aiming at the physicochemical and microbiological characterization of leaves and extract of C. ambrosioides. The toxicity of the extract was assessed using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) and acute toxicity was assayed in mice. The results showed that the leaves and the extract presented acceptable pharmacopeial parameters and the preliminary phytochemical analysis showed the presence mainly of saponins, terpenes, phenols and tannins in the hydroethanolic extract of C. ambrosioides. The microbiological evaluation of the extract indicated no apparent growth of pathogenic microorganisms. Although the extract showed cytotoxicity at high concentration, no signs of acute oral toxicity could be observed at the tested doses. These results, besides the partial Pharmacopeial characterization of C. ambrosioides, corroborate the literature data, regarding the cytotoxic potential. However, the results reported indicate that the use of this species is safe in appropriate doses.

INTRODUCTION: Medicinal plants are used in the treatment of diseases by thousands of people in the world and often represent the main and first source of health care. This form of therapeutic resource is accessible, available and culturally accepted in folk medicine.¹

In Brazil, a great percentage of the population makes use of natural products, mainly from plants, as an alternative source in the cultural context and as phytotherapics.² ³ The research interest in using medicinal plants has increased in recent years and among the factors driving this interest is the proven effectiveness of substances derived from plant species. Medicinal plants are important natural sources of biologically active substances, many of which are models for the synthesis of a large number of drugs, those products showing high diversity in structure and physicochemical and biological properties.⁴ ⁷
Noting the need of the market and the lack of quality control of many raw materials and extracts, this paper aims to study the hydroethanolic extract of the plant species Chenopodium ambrosioides Linn (Amaranthaceae), popularly known as “mstruz” and widely used in folk medicine, being considered one of the plant species with potential for use in the production chain and to generate products of interest to the Brazilian Unified Health System “Sistema Único de Saúde” (SUS), as part of the National List of Medicinal Plants of Interest to SUS (RENISUS) 8,9.

It is an annual or perennial herbaceous plant with distinctive aroma, upright, that reaches up to 1.5 m high, with dark green and camphorated pubescent leaves. Several therapeutic activities are assigned to the species, such as anti-inflammatory action, antifungal, antitumor, immunomodulatory, analgesic and antibacterial. The species is distinguished by its constitution rich in terpenes, flavonoids, gallic tannins and alkaloids 10-16. Although there are studies that attribute to C. ambrosioides a possible toxic action, allegedly resulting from the presence of the monoterpenic ascaridole, constituent abundant in the species 17-19, there are also reports of no toxicological changes in treating mice with different extracts from C. ambrosioides 20, 21. So, the relevance of research on the toxicity of the species using in vitro and in vivo models is highlighted.

MATERIALS AND METHODS:

Animals:
10 mice females (Mus musculus) were used, divided into two groups of five animals, weighing between 20g - 30g, from the Central Animal Facility of the Federal University of Pará (UFPA). During the adaptation period the animals were kept in the Animal Facility of the Faculty of Pharmacy for a week under standard conditions (temperature 22 ± 3°C, relative humidity 30-70% with control of light/dark cycle), as specified in the literature. The animals had access to food and water ad libitum. The methodology was approved by the Ethics Committee on Research with Experimental Animals, under the Protocol BIO098-12, taking into account "Ethical Principles in Animal Experimentation of the Brazilian College of Animal Experimentation."22.

Plant Material:
Leaves of C. ambrosioides used in this work were acquired in the Ver-o-Peso Market, coming from the Icoaraci district, municipality of Belém, located around the geographical coordinates of 1°10' and 1°20' south latitude and 48°20' and 48°30' west longitude. The identification of the plant sample was made at the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) and a voucher specimen of the species was deposited under registration number IAN188445 (Fig. 1).

FIG.1: VOUCHER SPECIMEN OF THE SPECIES CHENOPODIUM AMBROSIOIDES L. REGISTERED UNDER NUMBER IAN 188445

Obtaining the plant drug:
The fresh leaves were dried in an oven with circulating air at 40°C ± 2°C, until stabilizing the weight of an aliquot. After this condition grinding was executed in a Wiley mill (NOGUEIRA, Model DM JUNIOR). This process reduces the particle size of the sample and the powder obtained increases the contact surface between plant drug and fluid extractor.

Determination of particle size distribution:
Based on the Brazilian Pharmacopoeia (2010) and to analyze the grain size of the plant drug obtained from dried leaves of C. ambrosioides a sample of 25 g of the plant drug was subjected to vibration by
forced passage through sieves with mesh apertures corresponding to 125; 180; 250; 355; 710 and 1700 µm, using shaker sieves (Bertel) at the scale 10 of the apparatus, for thirty minutes. After this process, the plant drug retained in the sieves and collector was weighed. This procedure was performed in triplicate.

**Determination of loss from drying the plant drug**

1 g sample of plant drug were subjected to heating at 110°C by infrared rays for a period of approximately one hour. After this period weighing was done. The test was done in triplicate.

**Dry Matter Content:**

Aliquots of 2 ml of the extract were distributed evenly in an aluminum collector for later subjection to infrared moisture analyzer (Gehaka IV-2000) at a temperature of 105°C for fifteen minutes. The amount of moisture (or dry matter) was given in percentage, accompanied by the arithmetic mean and standard deviation of three determinations.

**Determination of pH:**

In accordance with the Brazilian Pharmacopoeia (2010), the pH of the extract was measured using a potentiometer (HANNA 58210) calibrated previously. Results were expressed as mean of three determinations.

**Determination of Ash Content**

For this test a porcelain crucible was previously calcined in a muffle furnace (ENGRO) for 30 minutes and then cooled in a desiccator for 15 minutes and weighed on an analytical balance. Aliquots of 3 g of plant drugs were distributed uniformly in the crucible and subjected to calcination in a muffle furnace at a temperature of 600°C for 180 minutes. The sample was allowed to cool in a desiccator for 30 min for later weighing. The results were expressed as percentage of the dry weight of drug ash (% w/w) representing the mean of three determinations.

**Preliminary Phytochemical Analysis:**

The hidroethanolic extract from *Chenopodium ambrosioides* Linn were subjected to preliminary phytochemical analysis aimed to detect different classes of secondary metabolites through chemical reactions, following the methodology described by Camelo and collaborators.

**Microbiological Quality Control:**

The total count of microorganisms and the possible detection of specific pathogens were performed using the methodologies described in the Brazilian Pharmacopoeia 5th Edition, by Pinto and collaborators (2010) and Silva Junior and colleagues (2011). Standard ATCC strains of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella* were used as positive controls.

**Cell Culture Assays for Cytotoxicity:**

Peritoneal macrophages from murine RAW 264.7 obtained from ATCC (Rockville, MD, USA) were kindly provided by the Laboratory of Natural Products Campus-Macaé, UFRJ (Federal University of Rio de Janeiro), and cultured at 37°C with 5% CO₂ in DMEM/F-12 medium supplemented with 10% fetal bovine serum and gentamicin (50 µg/mL).

**Evaluation of cell viability in RAW 264.7 macrophages:**

For the cytotoxicity assay the MTT method was used according to the procedure described by Mosmann (1983). RAW 264.7 macrophages grown in culture bottles were plated at a concentration of 1x10⁵ cells/well in a 96 well plate in the presence of the hydroethanolic extract of *Chenopodium ambrosioides* L. in concentrations of 0.8 µg/mL, 4 µg/mL, 20 µg/mL and 100 µg/mL in a final volume of 100 µL/well. In the wells used for negative control, macrophages were cultured in DMEM complete only without the extract. In the wells used as positive control, macrophages were treated with 1% Triton X-100. The plates were incubated for 24 hours at 37°C containing 5% CO₂. After this period, 10 µL of MTT (3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide) (Sigma) was added to each well.

After 2 hours of incubation at 37°C in the dark humid incubator, the supernatant of the plate was removed and the crystals formed were solubilized by 120 µL HCl (4 mM) in 80 mL of isopropanol (1N). Plates were read on a microplate reader using a 570 nm filter. Cytotoxicity was calculated using the following formula (O.D. – optical density):
Acutec Oral Toxicity Study (OECD, 2001): Each group of animals was kept fasting for 12 hours and subsequently received via a stomach tube, by gavage, the dose of 2500 mg/kg of extract C. ambrosioides L in 0.9% saline, the dose recommended by OECD for medicinal plants. Systematic behavioral observations were performed to evaluate the Hippocratic screening which provided a general estimate of the toxicity of the substance on the conscious state and general mood, activity and coordination of the motor system, reflexes and activities on the central nervous system and the autonomic nervous system. Parameters such as general activity, vocal tremors, irritability, touch response, response to tail clamping, corneal, tremors, convulsions, hair bristle, hypnosis, anesthesia, lacrimation, ptosis, urination, defecation, piloerection, hypothermia, breathing, cyanosis, hyperemia and death were determined in the 15 min, 30 min, 1 h, 2 h, 4 h and 8 h after administration and thereafter daily until the fourteenth day. The surviving animals were euthanized with a solution of xylazine (Bayer) and ketamine (Laboratory König SÁ) (2:1) at a dose of 2.5mL/kg and the organs were harvested for anatomo-histopathological evaluation.

Statistical Analysis: Data were analyzed using GraphPad Prism 4 software by analysis of one-way variance (ANOVA) followed by multiple comparison test (Tukey test) those results that showed “p” value < 0.05 were considered statistically significant.

RESULTS: The result of the sieve analysis of vegetable drugs shown in Fig.2, shows that the vegetable drug C. ambrosioides can be classified as coarse powder, since the entire sample went through the sieve of 1700 μm mesh and about 47% through the 355 μm mesh.

The percentage of dry crude extract C. ambrosioides L was 3.6% and preliminary data from the phytochemical analysis showed chemical groups that can be employed for the characterization of raw materials. This is the case of phenols, tannins, foam forming saponins, proteins and amino acids, triterpenoids and steroids (Table 2).
TABLE 2: DETERMINATION OF pH, DRY MATTER AND PHYTOCHEMICAL OF C. AMBROSIOIDES L.

<table>
<thead>
<tr>
<th>Determinations</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.60 ± 0.03%</td>
</tr>
<tr>
<td>Dry matter</td>
<td>3.6 ± 0.3%</td>
</tr>
<tr>
<td>Preliminary phytochemical analysis</td>
<td>Foam-forming saponins, proteins, amino acids,</td>
</tr>
<tr>
<td></td>
<td>phenols, tannins, steroids and terpenoids</td>
</tr>
</tbody>
</table>

Additionally, the microbiological quality control parameters not presented contamination by pathogenic microorganisms (Table 3).

TABLE 3: MICROBIOLOGICAL ANALYSIS OF THE HYDROETHANOLIC EXTRACT OF C. AMBROSIOIDES L.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bacteria (CFU/g)</th>
<th>Fungus (CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroethanolic extract of C. ambrosioides L.</td>
<td>Absence</td>
<td>Absence</td>
</tr>
<tr>
<td>ATCC control strains</td>
<td>&gt; 100 or Uncountable</td>
<td>&gt; 100 or Uncountable</td>
</tr>
</tbody>
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In the evaluation of cytotoxicity and acute oral toxicity of the hydroethanolic extract C. ambrosioides it was observed that the extract has a cytotoxic effect at the highest concentration used in this test, 100µg/ml (Fig.3). However, in the evaluation of acute oral toxicity, performed in animals treated with the extract at a dose of 2500 mg/kg, no signs or symptoms of alterations, such as compromise of central, autonomic or motor nervous system or behavioral changes were observed, considering the parameters evaluated and recommended by the OECD. Furthermore, no histopathological changes that correlate with toxicological findings in the organs of animals treated with saline (control group) were observed when compared with the group treated with the hydroethanolic extract of C. ambrosioides both as regards macroscopic or microscopic aspects, where the brain, lung, heart, kidney and liver were within normal histological limits (Fig. 4).

![Diagram](image_url)

FIG.3: PERCENT INHIBITION OF CELL VIABILITY OF MACROPHAGES STIMULATED WITH LPS AFTER TREATMENT WITH THE HYDROETHANOLIC EXTRACT OF CHENOPODIUM AMBROSIOIDES L. TRITON IS USED AS A POSITIVE CONTROL. THE DATA REPRESENT THE MEAN OF TRIPlicATES FROM ONE OF THREE EXPERIMENTS PERFORMED WITH SIMILAR RESULTS.

* p < 0.001 WHEN COMPARED TO THE NEGATIVE CONTROL (MACROPHAGES STIMULATED WITH LPS AND NOT TREATED WITH EXTRACTS - mØ + LPS).
DISCUSSION: Proper pulverization of vegetable raw material is of paramount importance to achieve a good performance in the extraction process of the chemical constituents contained in the plant drug. The particle size of the vegetable raw material is the parameter which determines the contact surface of the drug particles that interact with the solvent in obtaining pharmaceutical ingredients such as dyes and extracts. Thus, the particle size of herbal drugs directly affects the efficiency of the extraction process. The loss on drying of plant drug was 8.9% (Table 1), these results as well as those of Okhale and colleagues, who reported that moisture content of 10% for the drug from plant leaves C. ambrosioides are in accordance with the Brazilian Pharmacopoeia 5th Edition, which advocates maximum moisture content of up to 14% for herbal drugs.

The ash content for C. ambrosioides was 10%, the result is important for the quality standardization of herbal drugs, since a high ash content might indicate an excess of adherent substances, which may indicate inadequate collection, as well as the drying and transport of materials, or differences in geographic location of the materials analyzed. The percentage of dry crude extract C. ambrosioides L was 3.6% (Table 2). The dry matter is indicative of water content and volatile substances from the extraction of vegetable drugs, these data are important for calculating the yield of extraction, since the drying influences the integrity of cellular components, allowing these components to be exposed to contact with solvents, so this percentage corresponds to the concentration of the extract. The value of pH of the crude extract of C. ambrosioides is 6.6, which suggests the presence of weakly acidic compounds.

Microbiological quality control is essential to ensure the quality and safety of the product, especially when you have as raw material inputs of vegetable origin. The results presented here indicate that the material used for obtaining the hydroethanolic extract from the leaf of mazruz shows no growth of bacteria and fungus in the culture media used. The evaluation of cytotoxicity and acute oral toxicity of the hydroethanolic extract C. ambrosioides data are in agreement with those of literature showing cytotoxicity of the extract, essential oil, and single compounds of this species to eukaryotic cells, and demonstrated antitumor activity. However, this cytotoxicity does not implicated in significant toxicological activity in mice at the dose for studying acute oral toxicity.

CONCLUSION: The results presented contribute to the pharmacopeial characterization of derivatives of C. ambrosioides, providing inedited data, such as particle size determination and dry matter. Other data confirm the results already published that demonstrate the cytotoxic activity of the species,
moreover, they indicate that the hydroethanolic extract of *C. ambrosioides* presents no significant toxicological activity in mice at the dose recommended by OECD for studies of acute oral toxicity.

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